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Patterns of hydroxyproline excretion in ovariectomized and estrogen treated rats

Gregg Horras
University of Northern Iowa

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Gregg Horras

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Research advisor: Dr. Robert J. Simpson

Osteoporosis, a disease which is characterized by a decrease in bone mass that occurs with age, is a common problem among aging women, producing morbidity and mortality primarily through increased fracture rates. The burden that this disease presents to both families and the health care system is enormous. It has been estimated that osteoporosis causes at least 1.2 million fractures in the United States each year, and direct and indirect costs of the disease are estimated to be 6.1 billion dollars annually (1).

Osteoporosis has repeatedly been linked to the ovarian hormone deficiency brought about by either ovariectomy or menopause in both humans (1,2,3,4) and rats (5,6), and this induced osteopenia has been shown to be reversed by exogenous estrogen treatments in both humans (1,3,7) and rats (8,9,10). The mechanism by which estrogens affect bone has been debated. Several *in vivo* studies (5,8,9,10,11) in rats have suggested that ovariectomy speeds both bone formation and bone resorption, disrupting the already delicate balance between the two metabolic forces and causing osteopenia. Some of the same studies (8,9,10) also have suggested that estrogen treatments slow down the ovariectomy-induced increase in metabolic rate. In general, reports prior to 1988 discount the existence of direct effects of estrogen on bone and instead speculate that estrogens may have indirect effects which may be mediated through other hormones that are typically thought of as affecting bone metabolism, such as parathyroid hormone, calcitonin, or 1-25 dihydroxy vitamin D. However, a recent study has shown 17B-estradiol to have direct effects upon bone (12), and importantly, evidence of nuclear receptors for estrogens in osteoblasts have been reported (13,14).

If nuclear binding sites for estrogens exist in bone cells, then the origin of estrogen-deficiency-induced osteopenia may be at the level of

bone matrix protein synthesis. Since collagen is the most abundant protein in the bone matrix, and since hydroxyproline is an imino acid nearly exclusive to collagen, a monitoring of the metabolism of hydroxyproline in ovariectomized and estrogen treated rats may provide insight into the patterns of estrogen-deficiency-induced osteopenia. The purpose of our study was to test the hypothesis that ovariectomy will increase urinary hydroxyproline excretion in the rat and that this increase will be reversed by estrogen treatment therapy.

Materials and Methods

Animals - Twenty-six female Sprague-Dawley albino rats were purchased from Holtzmann. These rats were 31-34 days old upon arrival. The rats were housed in four large hanging cages (6-7 to a cage) on a rack with automatic watering fountains and automatic flush. The room was kept on 12-hr. dark, 12-hr. light cycles. The rats were provided Purina lab chow and water *ad libitum*.

Treatment schedule - Because only two metabolic cages were available for urine collections, experimental procedures on individual rats were staggered. On the first day that procedures were executed (27 days after the arrival of the rats), two rats which were labeled #1 and #2 were weighed, and their urine was collected overnight. On the following day, rats #1 and #2 were ovariectomized, and rats #3 and #4 were weighed and placed in urine collectors. On the following day, rats #5 and #6 were placed in urine collectors, and rats #3 and #4 were ovariectomized. This sequence was followed until all 24 rats had received surgeries and had given baseline urine samples. The order in which the 24 rats received treatments is outlined in the Appendix (table 1). After surgery each individual rat was independently housed. Injections were started for each

rat exactly one week after the initial surgery. The initial dosages of estrogen were ten times stronger than all subsequent dosages. After all rats had received initial injections, all rats then received injections every other day (all estrogen treated rats were injected on one day, and all vehicle treated rats were injected on the other day). All urine collections and injections were performed in the afternoon (between noon and 7 p.m., the exact time varied). After one round of collections was completed, the next round was generally initiated on the third day following, although after the penultimate collection round, an interval of 10 days passed before the next round (the final round) of collections was started. During the three days between collections 1 and 2 and between collections 2 and 3 injections were continued. However, during the 10 days between collections 3 and 4, no injections were given.

Note that each collection round was 12 days long. The ages of the rats during each collection were as follows:

Collection 1: 59-70 days

Collection 2: 88-99 days

Collection 3: 110-121 days

Experimental groups and treatments - Group I consisted of rats #1 through #8. These rats were ovariectomized and received vehicle injections. Group II consisted of rats #9 through #16. These rats were sham operated and received vehicle injections. Group III consisted of rats #17 through #24. These rats were ovariectomized and received estrogen injections. All injections were delivered subcutaneously at the back of the neck. Estrogen dosages were 10 ug/kg body weight. The concentration of the estrogen injections (with the exception of the first administration) were 15 ug/ml sesame oil. Vehicle injections were 0.15 ml. Surgeries were performed on rats which were anesthetized with ether. Urine was collected in metabolic cages for 17.5 to 24 hours, during which time the rats did not have access to food or water. Calculations for hydroxyproline

excretion were prorated to 24 hours for those urine samples which were collected for less than 24 hours. All urine collections made during collections 3 and 4 were for 24 hours ± 1/2 hour. Urine volumes were determined with a 10 ml graduated cylinder. Urine samples from collections 1, 3, and 4 were stored in a freezer. Urine samples from collection 2 were stored in a refrigerator and were spoiled by bacterial growth.

Statistical analysis- All statistical analyses (ANOVA, Scheffe's test) were calculated by a computer software program (SAS).

Urinary hydroxyproline assay- The hydroxyproline assay procedure utilized is based upon that of Bergman and Loxley (15). The oxidant solutions and the Ehrlich's reagent were prepared as recommended by their method. However, their procedure was not followed exactly but was modified as a result of a compilation of different assay procedures and independent observations. The procedure is described below.

1) *Hydrolysis and neutralization*. One ml of urine, one ml of deionized water, and one ml of 10N HCl were mixed in a 15 ml glass stoppered tube and heated for 24 hours in an oven at 105^0 C. When cool, the hydrolysate was transferred to a polyethylene centrifuge tube and centrifuged at 15000 rpm for 15 min. to remove solid material. The supernatant was then transferred to a 30 ml glass test tube, neutralized to phenolphthalein (two drops) with saturated lithium hydroxide (Sigma), made slightly acid again with 0.1 N HCl, diluted to 25 ml with water, covered and stored in a refrigerator until assay.

2) *Assay*. Chloramine T (the oxidant) and *p*-dimethylamino-benzaldehyde (Ehrlich's reagent) were purchased from Sigma. One ml of neutralized hydrolysate (or standard solution), two ml of isopropanol, and one ml of either oxidant solution or oxidant blank were mixed in a glass test tube. After four minutes, 2 ml of Ehrlich's reagent was added, and the tube was mixed. The absorbance at 560 nm was read 18 hours later.

Notes on the urinary hydroxyproline assay

The hydroxyproline assay utilized relies upon the successful oxidation of hydroxyproline to pyrrole with Chloramine T and the subsequent formation of a chromophore with *p*-dimethylaminobenzaldehyde. In preliminary experiments, the absorbance spectrum of the chromophore (see Appendix, figure 1) matched closely the absorbance spectrum presented originally (16), thus satisfactorily indicating the reproducibility of the procedure. Because over 95% of the hydroxyproline present in urine is found in a peptide-bound form (17), a hydrolysis step was added. Also, because solid material in the samples may cause light scattering during spectrophotometric analysis, a centrifugation step was added to remove precipitate. Lithium hydroxide was required to neutralize the hydrolyzates; it was noted in preliminary experiments that both KOH and NaOH will form precipitate with the Ehrlich's reagent solution. Also, the pH of the hydrolyzate must br carefully adjusted; a too highly acidic sample will deactivate the oxidant, and a highly basic hydrolyzate will result in the formation of precipitate.

By forming a similar oxidation product as hydoxyproline, other materials in the urine samples may form chrmophores as well. These interfering substances can be removed from consideration through the use of urine blanks, or non-oxidized urine samples. The absorbance of each urins sample is read against its own urine blank. However, in not one urine sample in our experiment did the blank absorb appreciably. Note, however, that the use of urine blanks does not eliminate the possibility of other substances that, without being oxidized, form chromophores with Ehrlich's reagent.

Standard hydroxyproline solutions used were 2.5 ppm, 5.0 ppm, 10 ppm, and 25 ppm. In all preliminary experiments, and for all experimental urine assays, the standard curve was very nearly linear. An example of a standard curve is shown in the Appendix (figure 2). This standard curve

was used in determining the urinary hydroxyproline concentrations from collection 1.

Results and Discussion

Collected hydroxyproline data is shown in table 1. Data from the third collection do indicate a significantly higher level of hydroxyproline excretion by the Group I rats (OVX+vehicle). For these rats, all three hydroxyproline parameters tabulated were statistically significantly higher than both the controls (Group II) and the estrogen treated rats (Group III). Also, in general, hydroxyproline excretion for the group III rats (OVX+E) was reduced as compared to the controls in the third collection. However, this reduction was statistically significant only when considering the parameter of urinary hydroxyproline concentration.

All of these observed trends did not continue into the fourth round. Rather, the collection from this round indicates slightly higher hydroxyproline excretion from the control (Group II) rats as compared to the ovariectomized, non-treated rats (Group I) as determined by all three hydroxyproline parameters tabulated. However, as was the case in collection 3, during collection 4 the estrogen treated rats (Group III) exhibited the lowest hydroxyproline excretion of all groups when considering all three hydroxyproline parameters tabulated. However, in only two cases were differences between groups statistically significant in collection 4; considering the parameters of urinary hydroxyproline concentration and total hydroxyproline excreted/body weight, data for group III rats was statistically significantly lower than data for both group II and group I rats.

Group II rats (OVX+E) gained weight at the greatest rate (figure 1), and group II rats (OVX+vehicle) gained weight at the slowest rate. The increased weight gain of ovariectomized rats is in concordance with the literature (16). However, the observation that the estrogen treated,

ovariectomized rats gained weight faster than the ovariectomized, non-treated rats suggests that ovariectomy induced weight gain may not be due to estrogen deficiency.

Timing was of crucial inportance in our experiments. The rats used were young, and their bones were rapidly growing, thus complicating the results. One laboratory (5) has measured femur growth in young rats and has reported rapid growth up to the age of 90 days, at approximately which time growth slows. Femur growth then stabilizes at 180 days. These observations have important implications for our results since all collections were made at times when the rats were near 90 days of age. A steady decrease was observed in all hydroxyproline parameters with time (table 1). This decrease was so pronounced that statistically significant differences were observed between collections in all measurement categories in all three groups. By the fourth collection, spectrophotometric readings were so low that they may not have been reliable.

Timing is an important consideration also because the urine collections were staggered. Groups II and III may have exhibited lower hydroxyproline measurements only because collections of group II and III rats were made later than those of group I. Because the collections were staggered, a urinary hydroxyproline vs. time curve could be constructed (figure 2). It is difficult to statistically ascertain from this plot whether or not the staggering did result in the significant lowering of the hydroxyproline concentrations of group II and III rats. It is unfortunate that this bias had to exist. It is necessary to remove this bias to conclude with certainty that the increased hydroxyproline excretion found in the ovariectomized, non-treated rats is due only to estrogen deficiency. This could most easily be accomplished by collecting urine from all experimental animals at the same time.

However, our studies do reinforce the notion that hydroxyproline excretion can be an effective means of monitoring bone metabolism. Since

increased hydroxyproline excretion was observed in younger, more rapidly growing rats, it is apparent that our data do generally correspond with the bone growth rate (and the concomitant bone turnover rate) in the rat. Other laboratories have produced similar findings; for example, it has been reported that urinary hydroxyproline excretion can be a reasonably precise and useful measure of resorption rate in postmenopausal women with osteoporosis (18). Also, urinary hydroxyproline/creatinine ratios have been found to be increased in ovariectomized women (19,20,21). Urinary hydroxyproline excretion in ovariectomized rats has not been previously reported. However, one very recent study has demonstrated that estradiol can depress urinary hydroxyproline excretion in intact, thyroidectomized, and parathyroidectomized rats (22).

The depression of hydroxyproline excretion in estrogen treated rats as compared to the control rats reflects the comparison between the exogenously derived estrogen levels and natural physiological estrogen levels. One laboratory (9) has reported that daily subcutaneous injections of estradiol at a dosage of 10 ug/kg B.W. resulted in serum estradiol levels that were significantly higher than normal physiological levels. In our study, rats were injected with the same dosage but on every other day. However, the fact that estrogen treated rats exhibited decreased hydroxyproline output suggests that this intermittent administration of estradiol may still have resulted in higher than physiological estradiol levels.

While not devoid of certain complications, our results do appear to generally reaffirm previously published results. In addition, we have provided previously undocumented information which can be used as reference for further experimentation. If further research into the hydroxyproline excretion of ovariectomized and estrogen treated rats were proposed, it would be recommended that 1) aged rats be used to more closely model the human pattern of postmenopausal bone metabolism and to avoid complications that are due to natural bone growth, and 2) all

treatments of all rats be carefully synchronized to avoid any age-related differences in hydroxyproline excretion between rats.

Table 1 Collected data. All values are mean ± S.D. For all groups, n=8. Values with the same letter are not statistically different from each other as determined by Scheffe's test (a,b,c: comparison between groups; d,e,f: comparison between collections). The P values are the result of an ANOVA run on each row and column of three means. V=vehicle, E=estradiol.

		Groups			
Parameter	Collection	I (OVX+V)	II (Sham+V)	III (OVX+E)	P
total hyp excreted (ug)	1	415±185^{a,d}	292±118^{a,d}	347±104^{a,d}	0.2376
	3	342±67^{a,d}	204±62^{b,d,e}	183±55^{b,e}	0.0001
	4	109±48^{a,e}	120±56^{a,e}	66±37^{a,f}	0.0786
	P	0.0001	0.0020	0.0001	
urinary hyp conc. (ug/ml)	1	77.5±22.6^{a,d}	75.1±15.0^{a,d}	73.1±15.2^{a,d}	0.8854
	3	80.9±14.0^{a,d}	56.5±18.3^{b,d}	37.6±8.2^{c,e}	0.0001
	4	22.4±7.4^{a,b,e}	26.6±11.3^{a,e}	13.9±6.6^{b,f}	0.0239
	P	0.0001	0.0001	0.0001	
tot. hyp excreted/B.W. (ug/kg)	1	1860±770^{a,d}	1300±510^{a,d}	1510±460^{a,d}	0.1979
	3	1220±250^{a,e}	773±257^{b,e}	661±182^{b,e}	0.0002
	4	315±126^{a,b,f}	410±190^{a,e}	212±109^{b,f}	0.0430
	P	0.0001	0.0002	0.0001	

Figure 1 Rat mean body weights

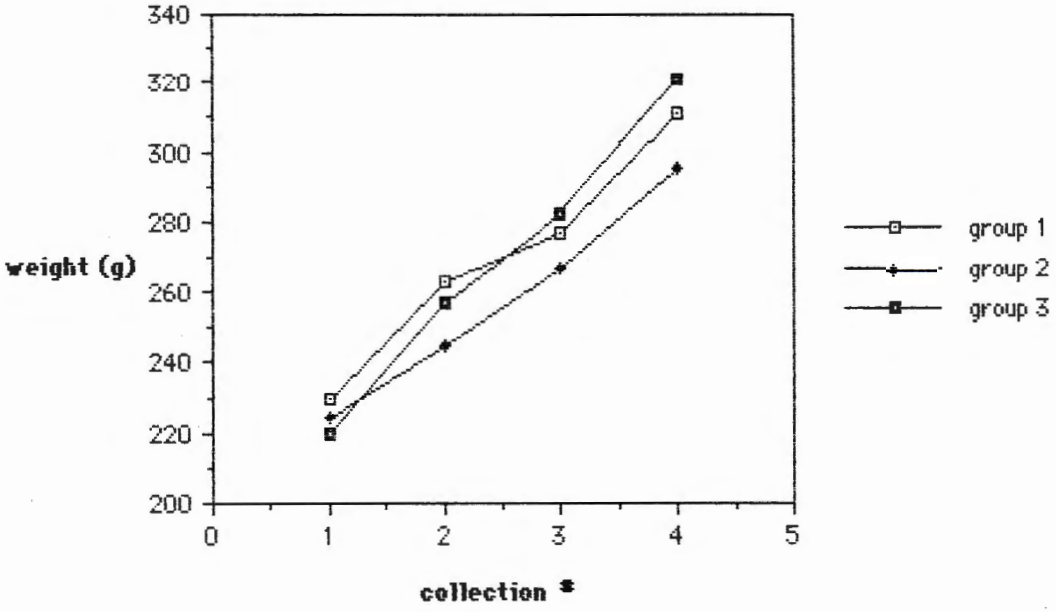
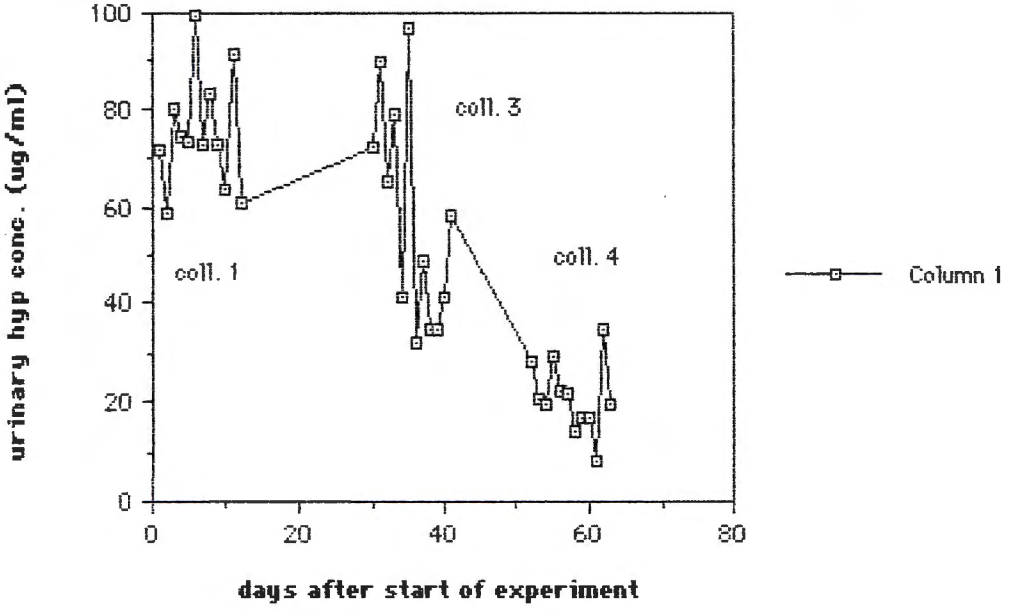


Figure 2 Urinary hydroxyproline excretion vs. time.



Appendix

Table 1 The collection schedule.

Day of collection cycle	Rats from which urine was collected	Group
1	1,2	I
2	3,4	I
3	5,6	I
4	9,10	II
5	11,12	II
6	7,8	I
7	17,18	III
8	19,20	III
9	21,22	III
10	23,24	III
11	13,14	II
12	15,16	II

Figure 1 Absorbance spectrum of hydroxyproline (10 ppm standard, 14 hr.)

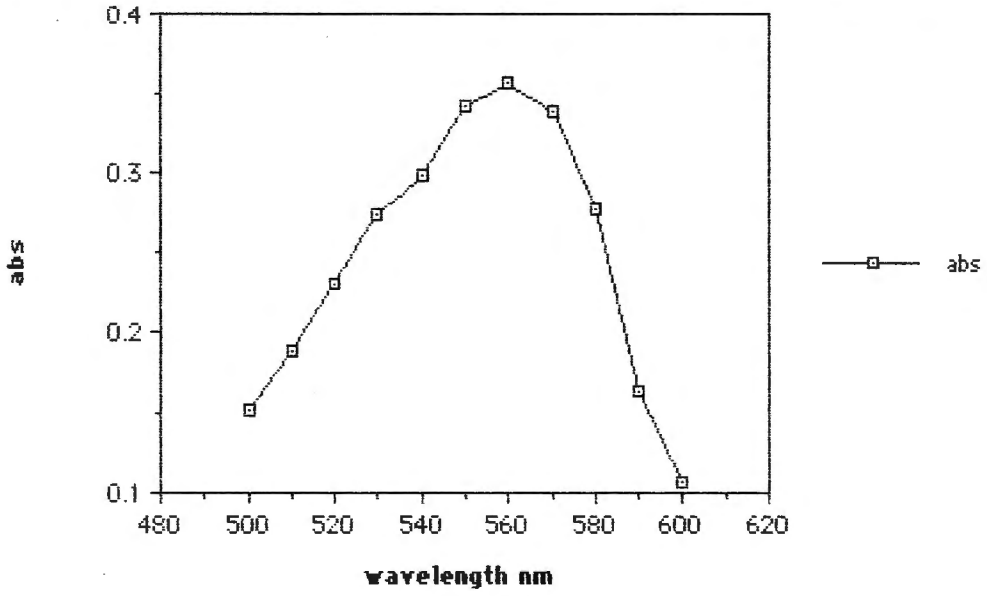
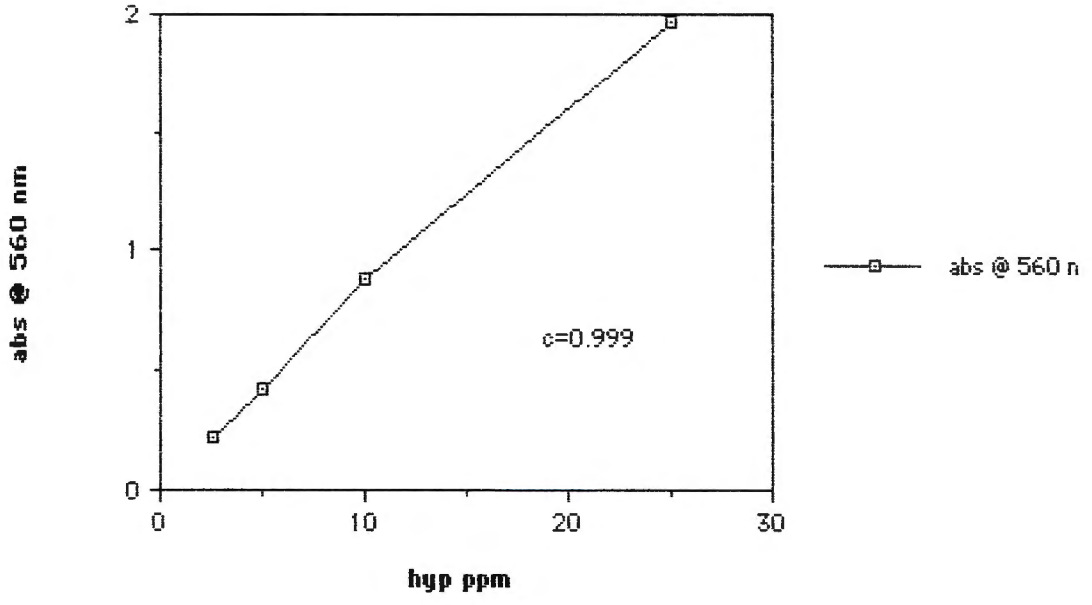


Figure 2 Hydroxyproline standard curve.



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